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ORIGINAL ARTICLE

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Clinical pharmacokinetics of the irinotecan metabolite 4-piperidinopiperidine and its possible clinical importance

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Abstract *Purpose*: To investigate the clinical relevance of 4-piperidinopiperidine (4PP) in the activity of irinotecan (CPT-11), a high-performance liquid chromatography-turboionspray-tandem mass spectrometry assay for plasma 4PP was developed. *Methods*: Plasma samples were prepared for analysis following C18 solid-phase extraction. Chromatography was performed on a Waters Nova-Pak Phenyl column. Selected reaction monitoring with the mass transitions m/z 169.2 → 84.2 and 139.2 → 98.1 was used for the detection of 4PP and the internal standard (IS), 1-piperidineproprionitrile, respectively. *Results*: The assay was linear from 14.8 to 591.0 n*M* with absolute recoveries of 4PP (59.1 n*M*) and IS (143.7 n*M*) of 85.7% ($n = 10$) and 86.7% ($n = 10$), respectively. The accuracy and imprecision of the method (total) was ≥96.8% and ≤8.5% over the concentration range studied, respectively. 4PP was detectable in plasma following the administration of 125, 350, 500 mg/m^2 and 600 mg/m^2 CPT-11 to patients, with AUC_{4PP} correlated with the dose ($r^2 = 0.66$). Plasma concentrations of 4PP declined slowly with a long terminal half-life (33.4 ± 17.1 h). *Conclusions*: Overall, the concentrations of 4PP in plasma were in the sub-micromolar range (<206.9 n*M*) and substantially lower than those capable of inducing apoptosis of cancer cells.

Key words 4PP · CPT-11 · High-performance liquid chromatography · Turboionspray · Tandem mass spectrometry

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Introduction

Irinotecan (CPT-11; Campto; Camptosar), an analogue of the alkaloid camptothecin, is currently registered for the treatment of advanced colorectal adenocarcinoma not responding to fluoropyrimidines [1]. Camptothecins exert their anti-tumour effects by stabilizing ternary complexes which form between the nuclear enzyme topoisomerase I (topo I) and DNA. These lead to the formation of double-stranded DNA breaks and, consequently, apoptosis (reviewed in ref [2]). In vivo, CPT-11 is converted to the active metabolite, SN-38, which is 100–1000 times more potent than CPT-11 in its interaction with topo I in vitro. Hence, SN-38 is thought to account for most of the anti-tumour activity of CPT-11 [3, 4, 5].

However, several recent studies have indicated that other additional mechanisms could be involved in the anti-tumour activity of CPT-11. For example, there is some evidence that CPT-11 may have anti-angiogenic properties [6]. In addition, it has been reported that 4-piperidinopiperidine (4PP), which is released during the esterolysis of CPT-11 to SN-38 (Fig. 1) is able to induce apoptosis in a lymphoma cell line (RVC) in vitro [7]. Furthermore, cells selected for resistance to 4PP by continuous exposure to this compound developed cross-resistance to CPT-11, indicating the presence of a potentially novel mechanism.

Although the concentrations required to induce apoptosis were relatively high (300 μ*M* and 30 μ*M* in RVC and mouse thymocytes, respectively), it is unknown how these relate to the clinical situation, because 4PP has

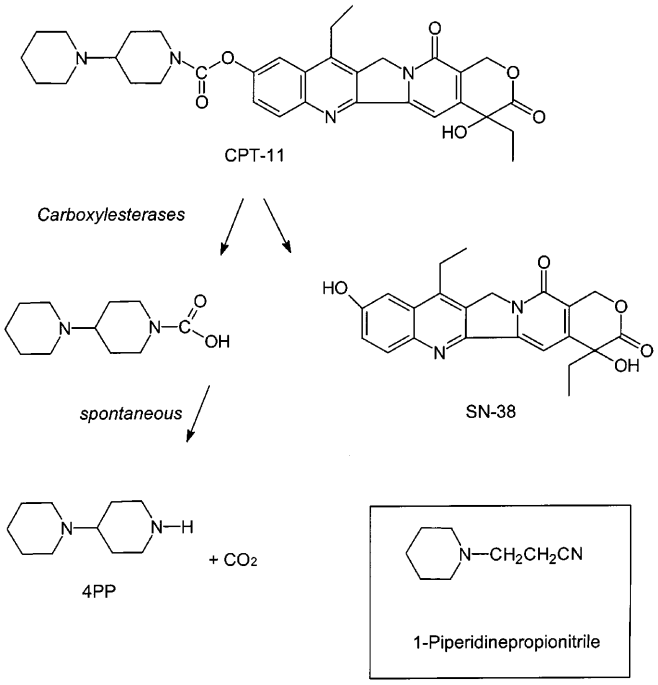

Fig. 1 The chemical structures of CPT-11, SN-38, 4PP and 1-piperidinepropionitrile (internal standard; IS). *CPT-11* irinotecan, *4PP* 4-piperidinopiperidine

been overlooked in pharmacokinetic studies carried out to date. Therefore, the aim of the current study was to determine the concentrations of 4PP in the plasma of patients administered CPT-11 at 125-, 350-, 500-mg/m^2 and 600-mg/m^2 doses, either in weekly (125 mg/m^2) or every-3-week regimens (350, 500 mg/m^{2}and 600 mg/m^2).

Given that the conversion of CPT-11 to SN-38 by human liver carboxylesterase in vivo is relatively inefficient and the concentrations of SN-38 are modest, we anticipated that plasma concentrations of 4PP would most likely fall in the low micromolar range. On this basis, a sensitive, robust method was required.

This study reports the validation of the assay employed to detect the concentrations of 4PP in human plasma by high-performance liquid chromatography-turboionspray-tandem mass spectrometry (HPLC-TIS-MS/MS). We illustrate its applicability by presenting the plasma pharmacokinetics of 4PP in patients treated with CPT-11 over a range of doses.

Materials and methods

Chemicals and HPLC reagents

CPT-11 was obtained from Rhône-Poulenc Rorer (Neuilly, France). 4-Piperidinopiperidine and 1-piperidinepropionitrile (internal standard; IS) were purchased from Sigma-Aldrich Pty Limited (Sydney, Australia). Figure 1 illustrates their chemical structures. Water was of Milli-Q grade (Millipore, Brisbane, Australia) and all other chemicals were of analytical grade or above. All HPLC solvents were filtered and degassed using a 0.45-μm filtration system (Millipore). All validation studies used plasma obtained from patients of the Princess Alexandra Hospital not receiving chemotherapy.

Apparatus

The HPLC system consisted of a 616 solvent delivery system with a 600S controller and a 710B WISP autoinjector (Waters, Milford, Mass., USA). Chromatography was achieved using a Waters Nova-Pak Phenyl column (2 × 150 mm, 4 μm) at ambient temperature. The mobile phase consisted of a mixture (80/20, v/v) of acetonitrile (ACN) and 40 m*M* ammonium acetate buffer (pH 4.0) delivered at a flow rate of 300 μl/min. Mass spectrometric detection was performed on an API III triple quadrupole instrument (PE-Sciex, Thornhill, Toronto, Canada) using selected reaction monitoring (SRM). A turboionspray (TIS) interface was used in positive ionization mode. The orifice potential, probe and interface temperatures were set to 60 V, 550 °C and 60 °C, respectively, to produce protonated species of the analytes. For collision-activated dissociation, argon was used at a thickness of 300×10^{12} molecules cm^{-2}. Peak area ratios obtained from SRM of 4PP (m/z 169.2 → 84.2) and IS (m/z 139.2 → 98.1) were used for quantification. Standard curves (14.8–591.0 n*M*) were constructed using weighted ($1/x^2$) linear least-squares regression. Data were collected and analysed on MacIntosh computers operating RAD and MACQUAN software (Pe-Sciex).

Sample preparation

Stock solutions of 4PP (5.9 m*M*) and IS (7.2 m*M*) were prepared in water. These solutions were then diluted further in water to give working solutions of 5.9 μ*M* and 1.4 μ*M*, respectively. From the 4PP working solution, dilutions in plasma were performed to give a series of working standards (14.8, 29.6, 59.1, 118.2, 295.5, 472.8 n*M* and 591.0 n*M*) and controls (17.7, 44.3 n*M* and 443.3 n*M*). A further solution of 118.2 n*M* was also prepared from the 4PP working stock for stability studies in plasma.

Standards, controls and patient samples (1 ml) were treated with 1 ml water, containing 20 ng of IS, in 10-ml polypropylene tubes (Sarstedt, Adelaide, Australia). Samples were vortex-mixed for 1 min and centrifuged at 850 *g* for 5 min. The supernatants were applied to 100 mg C18 solid-phase extraction cartridges (Waters), which had been pre-conditioned with methanol (3 ml) and water (3 ml). The loaded cartridges were rinsed with 1 ml water and placed under full vacuum for 15 min. The analytes were eluted with the mobile phase (1 × 50 μl, 1 × 200 μl) into Eppendorf tubes. The eluant was centrifuged at 8000 *g* for 5 min and 10 μl injected onto the chromatogram. In some cases, the concentrations of 4PP were slightly lower than the analytical range and 20 μl of the eluant was re-injected onto the chromatogram.

Validation of HPLC-TIS-MS/MS assay

Standard curves and controls were prepared and assayed daily. The specificity of the assay was assessed by assaying plasma taken from patients not on drug therapy. Linearity was confirmed by plotting the ratio of 4PP to IS peak areas versus drug concentration ($n = 10$). The method's accuracy and inter-day imprecision over the analytical range were determined by back-calculated results of the linearity study. The imprecision of the assay was also determined by assaying the plasma controls by replicate analysis ($n = 4$) on each of 4 days. Intra-day, inter-day and total imprecision were derived from analyses of variance of the assayed controls using the method of Krouwer and Rabinowitz [8]. Accuracy was determined by expressing the mean assayed result for the control samples ($n = 16$) as a percentage of the known concentration. Absolute recovery of the analytes was determined by comparing the peak areas of samples spiked with 4PP (59.1 n*M*) and IS (143.7 n*M*) before and after extraction ($n = 10$).

Stability samples (1 ml) were prepared in sterile 10-ml polypropylene tubes and stored at room temperature for 0, 1, 2 h and

4 h before analysis. Samples were prepared identically and stored at 4 °C, −20 °C and −70 °C for analysis on days 3, 7, 11, 14, 20 and 48. In addition, some of these samples were then re-assayed the following day to assess the stability of 4PP in mobile phase.

Pharmacokinetic studies of 4PP

The concentrations of 4PP in the plasma of patients treated with CPT-11 (125, 350, 500 mg/m^2 or 600 mg/m^2) were determined following the collection of 10 ml venous blood into heparinized tubes. The samples were centrifuged immediately and the plasma stored at −70 °C until analysis. Samples were collected into lithium heparin tubes at pre-dose, end of infusion, 5, 10, 15, 20, 30, 45 min and 60 min and 1.5, 2, 4, 6, 8, 12, 18 h and 24 h after infusion of CPT-11. Patients already recruited into a study for the compassionate use of CPT-11 in advanced colorectal adenocarcinoma, not responding to 5-fluorouracil, were invited to participate in this investigation. This study was approved by the Ethics Committees of the Royal Prince Alfred and Concord Hospitals. Written informed consent was obtained from each patient. Samples collected during phase I/II trials in France were also included.

Analysis of total CPT-11, APC, SN-38 and its glucuronide

Plasma concentrations of total CPT-11 (lactone + carboxylate) and known metabolites APC and SN-38 were determined using a modification of the method described by Rivory and Robert [9]. The plasma concentrations of the glucuronide conjugate of SN-38 was determined following pretreatment with β-glucuronidase as per Rivory and Robert [10]. Standard samples were prepared from stock solutions diluted serially into an aqueous solution of ACN: water (50:50, v/v). Separation was carried out using a Waters C18 Nova-Pak Radial-Pak column (5 × 250 mm, 4 μm) at ambient temperature and with a solvent flow of 1.5 ml/min. The mobile phase consisted of ACN and 0.075 *M* ammonium acetate buffer (pH 4.5) mixed with a solvent selection valve in the proportion of 22:78 (v/v). Fluorescence detection (RF-10AXL, Shimadzu, Sydney, Australia) was optimized for detection of SN-38 with excitation and emission wavelengths set at 380 nm and 530 nm, respectively. Data were collected and analysed using CLASS VP software (version 4.2, Shimadzu). Using this assay, the lower limits of quantification of total (lactone + carboxylate) CPT-11, APC and SN-38 were 15, 8 n*M* and 1 n*M*, respectively.

Pharmacokinetic analysis

The areas under the concentration-time curve (AUCs) of the compounds of interest were calculated using the trapezoidal method and extrapolated to infinity using the terminal rate constant estimated from a regression of the linear semi-log concentration versus time profile at later time points. The terminal half-life of elimination ($t_{1/2z}$) was estimated as 0.693 divided by the terminal rate constant.

Results

The use of a low orifice potential (60 V) was employed to produce the pseudomolecular ion $[M + H]^+$ of 4PP (m/z 169.2) and the internal standard, 1-piperidinepropionitrile (m/z 139.2). The predominant daughter ion of each compound (m/z 84.2 and 98.1, respectively) was selected and optimized for SRM.

The assay was linear over the range 14.8 to 591.0 n*M* ($r \geq 0.997$, $n = 10$), with a lower limit of detection of 5.9 n*M*. Accuracy and imprecision over the analytical range were 95.8–100.8% and 2.9–5.2%, respectively (Table 1). Table 2 depicts the accuracy, intra-, inter-day and total coefficients of variation of 4PP at concentrations of 17.7, 44.3 n*M* and 443.3 n*M*. The accuracy and imprecision at these concentrations were 96.8–99.0% and ≤8.5%, respectively. Absolute mean recoveries for 4PP (59.1 n*M*) and IS (143.7 n*M*) were determined to be 85.7% ($n = 10$) and 86.7% ($n = 10$), respectively. The evaluation of the stability of 4PP in plasma stored under several conditions was also conducted. As shown in Table 3, no degradation or loss of 4PP was evident under all conditions investigated over a 48-day period.

In addition, the stability of 4PP in mobile phase at ambient temperature was assessed at a concentration of 118.2 n*M*. After 24 h of storage, the estimated concentrations ($n = 10$) were 101.3% of initial with a CV of 2.9%.

The maximal plasma concentration (C_{max}) and AUC of 4PP was shown to increase with the dose of CPT-11

Table 1 Linearity, accuracy and inter-day imprecision over the analytical range, *4PP* 4-piperidinopiperidine

Day	r^2	4PP concentration (n*M*)						
		14.8	29.6	59.1	118.2	295.5	472.8	591.0
1	0.997	14.95	30.14	55.55	109.93	278.34	497.64	625.89
2	0.997	14.59	31.03	59.10	104.02	304.96	508.27	578.01
3	0.998	15.13	28.90	57.33	111.70	316.19	449.17	614.07
4	0.998	14.89	28.90	38.42	111.11	291.37	504.73	597.52
5	0.998	15.48	30.32	36.64	124.11	302.60	462.77	605.79
6	0.999	14.48	26.95	60.28	119.39	309.69	485.82	573.80
7	0.998	14.78	30.67	54.96	112.88	316.19	488.77	576.24
8	0.997	13.89	31.32	57.92	112.88	294.33	457.45	601.06
9	0.997	14.07	32.03	62.06	108.16	272.46	469.86	593.38
10	0.999	14.48	31.09	57.92	118.79	290.78	471.63	589.24
Mean	–	14.66	30.08	57.33	113.48	297.87	479.31	595.74
Accuracy (%)	–	99.3	102.0	97.4	95.8	100.7	101.4	100.8
Inter-day imprecision	–	3.3	5.0	4.1	5.2	5.1	4.3	2.9

Correlation coefficient was determined by weighted ($1/x^2$) linear least-squares regression. Accuracy was determined as a (%) of the mean assayed concentration over the weighed-in concentration. Inter-day imprecision is expressed in terms of (%) coefficient of variation

Table 2 Imprecision and accuracy of the determination of 4PP in human plasma during a 4-day validation ($n = 4$ each day), using control samples. *4PP* 4-piperidinopiperidine

4PP concentration (n*M*)	Accuracy (%)	Imprecision (%)[a]		
		Within-day	Between-day	Total
17.7	98.95	6.40	3.18	7.15
44.3	98.52	5.64	6.35	8.50
443.3	96.89	2.27	0.75	2.39

[a] Expressed as the coefficient of variation calculated by the method of Krouwer and Rabinowtz [8]

(Table 4, see Fig. 2). However, as shown in Fig. 3, the AUC_{4PP} was poorly correlated with $AUC_{CPT\text{-}11}$ ($r^2 = 0.41$). The plasma concentrations of 4PP peaked at varying times (0.78 ± 0.45 h, Table 4). In comparison, maximal concentrations of SN-38 occurred earlier ($C_{max} = 0.38 \pm 0.46$ h, Table 4) and usually coincided with the end of infusion, particularly at the lower doses (Table 4). Concentrations of 4PP decreased very slowly over the sampling time range (Fig. 4). Although this precluded accurate assessment of $t_{1/2z}$, particularly for the lowest dose of CPT-11 (125 mg/m^2), estimates are shown in Table 4. For comparative purposes, the pharmacokinetics of total CPT-11, APC, SN-38 and its glucuronide are also included in Table 4.

Discussion

This study is the first to examine the pharmacology of 4PP in patients treated with CPT-11. It has been shown previously that the plasma kinetics of CPT-11 and SN-38 display wide inter-individual variation and this is most likely to be due to the extensive inter-patient differences in the metabolic pathways of CPT-11 [11, 12]. Indeed, glucuronidation of SN-38 has been shown to vary considerably with the responsible isoform of glucuronosyl-transferase known to be the subject of pharmacogenetic differences [13].

For each dose regimen, the concentration-time profile for 4PP revealed maximal concentrations within the first hour post-infusion (mean = 0.78 h). Plasma concentrations of 4PP then declined very slowly with a long

Table 3 Stability of 4PP (118.2 n*M*) in human plasma at ambient temperature (23 ± 1 °C, $n = 16$), at 4, −20 °C and −70 °C ($n = 20$ for each) expressed as a percentage of theoretical concentration. *4PP* 4-piperidinopiperidine, *CV* coefficient of variation

Day	Time (h)	Stability 23 °C	CV (%)	Stability 4 °C	CV (%)	Stability −20 °C	CV (%)	Stability −70 °C	CV (%)
1	0	83.4	1.4						
1	1	94.2	0.97						
1	2	94.1	1.4						
1	4	95.4	0.69						
3				100.5	1.1	98.3	1.5	89.7	0.55
7				90.6	0.83	91.1	0.71	96.0	0.72
14				100.3	0.63	100.0	0.43	100.5	0.99
20				116.9	1.5	106.8	1.7	109.9	1.4
48				106.5	0.77	112.7	1.9	123.7	0.6

Table 4 Pharmacokinetic parameters of total CPT-11 (mean ± SD) and 4PP, total APC, total SN-38 and SN-38 glucuronide as a function of CPT-11 dose (mean with range in parentheses). *CPT*-11 irinotecan, *4PP* 4-piperidinopiperidine, *AUC* area under the concentration-time curve, C_{max} maximal plasma concentration, $t_{1/2z}$ terminal half-life of elimination

Metabolite	Parameter	CPT-11 dose (mg/m^2)			
		125 ($n = 4$)	350 ($n = 5$)	500 ($n = 2$)	600 $n = 2$
4PP	AUC (μ*M* · h)	1.0 (0.35–2.11)*	2.96 (1.77–4.07)	4.58 (4.54–4.62)	3.6**
	C_{max} (μ*M*)	0.019 (0.012–0.035)	0.086 (0.06–0.13)	0.121 (0.098, 0.143)	0.162 (0.136, 0.187)
	$t_{1/2z}$ (h)	37.8 (15.0–72.0)*	32.0 (23.2–47.9)	39.6 (28.6, 50.6)	14.64**
APC	AUC (μ*M* · h)	3.1 (2.0–4.1)	9.6 (6.8–12.8)	26.1 (22.5, 29.8)	24.0 (19.7, 28.4)
	C_{max} (μ*M*)	0.16 (0.13–0.21)	0.88 (0.62–1.13)	1.25 (1.23, 1.28)	1.84 (1.54, 2.13)
	$t_{1/2z}$ (h)	10.5 (4.0–18.2)	10.4 (7.4–12.3)	11.2 (9.7, 12.8)	9.2 (8.5, 9.8)
SN-38	AUC (μ*M* · h)	1.5 (0.84–2.1)	3.5 (1.7–5.8)	6.3 (4.7, 8.0)	6.2 (4.1, 8.3)
	C_{max} (μ*M*)	0.17 (0.11–0.31)	0.57 (0.46–0.88)	0.62 (0.57, 0.67)	0.84 (0.69, 0.99)
	$t_{1/2z}$ (h)	13.1 (4.9–33.3)	26.0 (16.1–37.5)	14.8 (10.9, 18.7)	9.5 (8.9, 10.2)
SN-38G	AUC (μ*M* · h)	4.4 (1.6–8.0)	6.9 (4.4–12.5)	20.8 (15.8, 25.7)	12.2**
	C_{max} (μ*M*)	0.31 (0.20–0.44)	0.76 (0.55–1.01)	1.43 (1.41, 1.47)	0.54**
	$t_{1/2z}$ (h)	11.5 (6.5–18.8)	13.5 (7.0–20.7)	19.0 (16.2, 21.9)	24.4**
CPT-11	AUC (μ*M* · h)	10.2 ± 5.8	27.4 ± 9.3	48.9 ± 3.3	55.8 ± 16.1
	C_{max} (μ*M*)	1.7 ± 0.7	12.7 ± 7.0	9.8 ± 0.2	13.7 ± 0.6
	$t_{1/2z}$ (h)	5.2 (3.7–7.8)	8.9 (6.0–11.6)	9.9 (7.5, 12.4)	9.5 (6.9, 12.1)

* $n = 3$ due to insufficient volume of sample for analysis
** $n = 1$ due to insufficient volume of sample for analysis

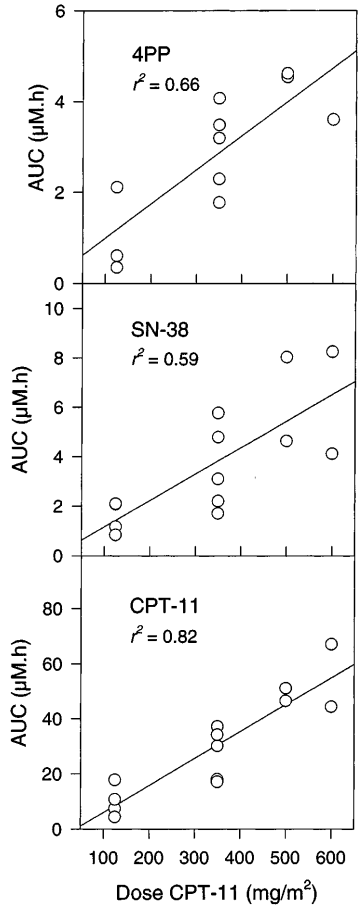

Fig. 2 The relationship between the AUCs of each of the compounds of interest as a function of the dose of CPT-11. *AUC* area under the concentration-time curve, *CPT-11* irinotecan

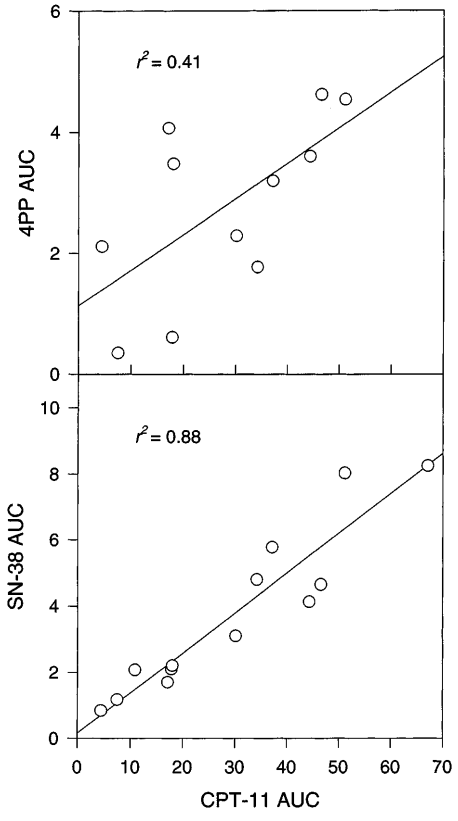

Fig. 3 The relationship between the AUCs of CPT-11, 4PP and SN-38. *CPT-11* irinotecan, *4PP* 4-piperidinopiperidine, *AUC* area under the concentration-time curve

terminal half-life of 33.4 ± 17.1 h. In this preliminary investigation, plasma samples were only collected up to 24 h and this was insufficient to fully characterize this late phase. Further studies will be required to evaluate the late kinetics of 4PP and whether accumulation of 4PP occurs on the different protocols (weekly, every 3 weeks).

The concentration-time profile for SN-38 did not parallel that of 4PP. Plasma concentrations of SN-38 were initially five- to ten-fold higher than corresponding 4PP concentrations but the plasma decay of SN-38 was more rapid ($t_{1/2z}$ = 19.3 ± 11.7 h, see Fig. 4). Indeed, in most instances, the concentrations of 4PP were equal to or greater than SN-38 towards the end of the course of sampling (Fig. 4).

The recent finding of Onishi et al. [7] in which 4PP induced apoptosis in a lymphoma cell line needs to be re-examined in the light of our results. They reported an IC_{50} for the RVC cell line of approximately 600 μ*M* when the cells were exposed to 4PP over 24 h. This corresponds to an estimated AUC_{4PP} of 21,300 μ*M* · h which is significantly greater than those observed (0.35–4.62 μ*M* · h) over the range of doses given (125–600 mg/m^2). Onishi et al. [7] reported some growth inhibition

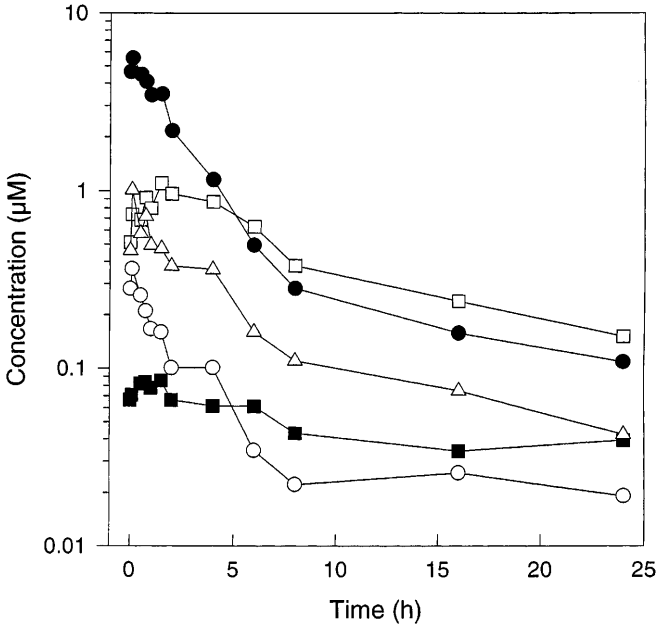

Fig. 4 Plasma concentrations of total CPT-11 (*closed circle*), 4PP (*closed square*), total APC (*open square*), total SN-38 (*open circle*) and SN-38G (*open triangle*) in a patient receiving 350 mg/m^2 of CPT-11. *CPT-11* irinotecan, *4PP* 4-piperidinopiperidine

(cf. apoptosis) at lower concentrations (approximately 150 μ*M*). Nevertheless, even these latter concentrations are far in excess of those measured in plasma (approximately 1000-fold). Alternatively, the persisting concentrations of 4PP observed in our study could be expected to lead to selection of cells resistant to 4PP and perhaps to CPT-11 as demonstrated by Onishi et al. [7]. However, tolerance to 3 m*M* 4PP lead only to a six-fold resistance to CPT-11 in the RVC line [7]. Therefore, it is unlikely that 4PP contributes significantly to the anti-cancer activity of CPT-11 in vivo.

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